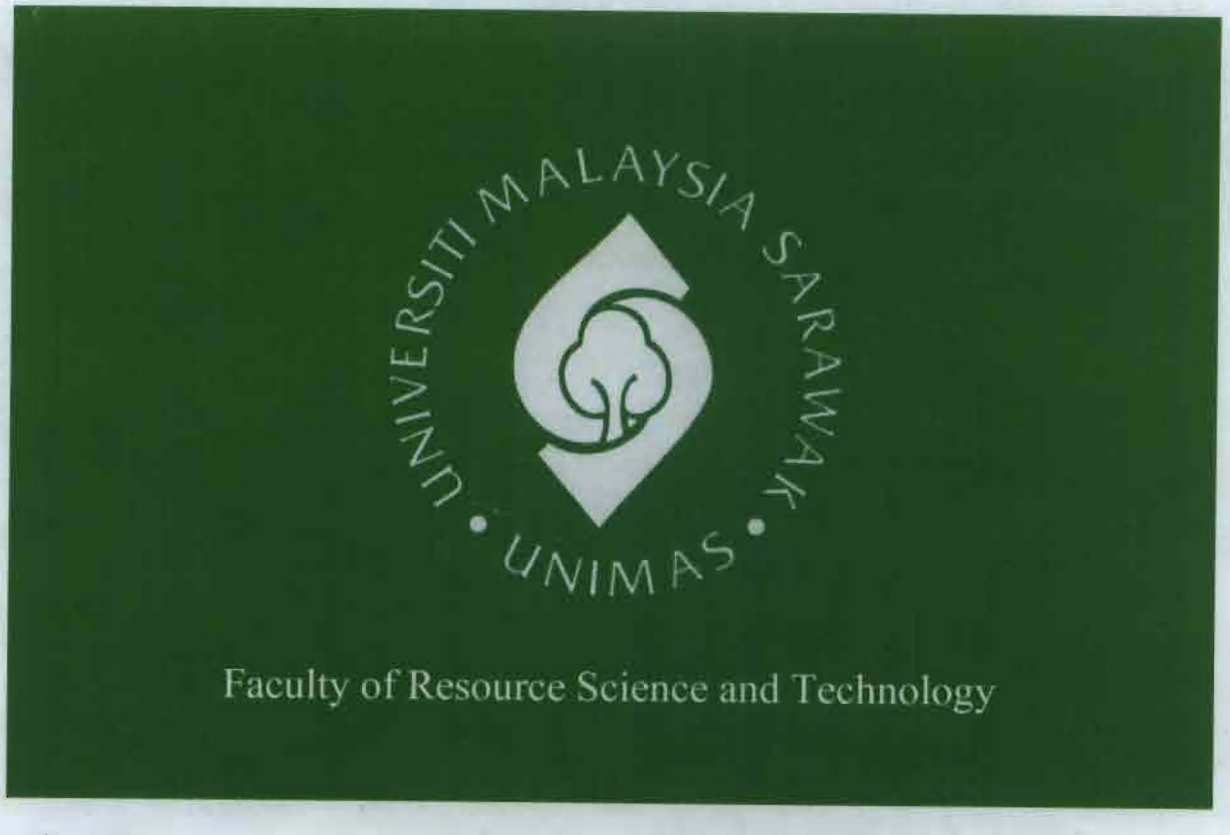

ISOLATION OF HETEROCYCLIC HYDROCARBONS FROM SEAWATER

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Isolation of Heterocyclic Hydrocarbons from Seawater

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A final project report submitted in partial fulfillment of the Final Year Project II (STF 3015) course

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Declaration

I declare that this thesis entitled "Isolation of Heterocyclic Hydrocarbons from Seawater" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : .....

Name : .....

Date : .....

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List of Abbreviations

L	Liter
mL	Mililiter
µl	Microliter
g	Gram
°C	Degree Celsius
rpm	Round per Minute
16S rDNA	16S ribosomal DNA
16S rRNA	16S ribosomal RNA
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
DNA	Deoxyribonucleic Acid
MR-VP	Methy Red-Voges Proskauer
CTAB	Cetyl Trimethyl Ammonium Bromide Cationic
SDS	Sodium Dodecyl Sulfate
TE	Tris EDTA
UV	Ultraviolet

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Isolation of Heterocyclic Hydrocarbons from Seawater

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Abstract

Heterocyclic hydrocarbons are widely distributed in soils and sediments, groundwater, atmosphere, and marine environment. They are very stable organic compounds that persist in environment for long period of time. The presence of heterocyclic hydrocarbon in the seawater due to the industrial process and spillage of petroleum causes the pollution to the marine environment and may affect human health. Bioremediation is one of the solutions to degrade the heterocyclic hydrocarbon. It involves the activity of microorganisms that oxidizes the aromatic ring of the heterocyclic hydrocarbon and degrades it. The degrading bacteria that isolated from the seawater are useful to clean up the heterocyclic hydrocarbon in seawater. This study aims to isolate heterocyclic hydrocarbon degrading bacteria from the coast of Sarawak. Five bacteria were isolated and characterized via biochemical tests and molecular technique. The degradation ability of the isolated bacteria was determined by testing the chemical properties for each bacterium. The result shows that the isolated bacteria may play some role in heterocyclic hydrocarbon degradation.

Keywords: Heterocyclic hydrocarbon, bioremediation, degrading bacteria, marine environment.

Abstrak

Hidrokarbon heterosiklik terdapat secara meluas di alam sekitar seperti tanah dan sedimen, air bawah tanah, dan persekitaran marin. Mereka adalah organik yang sangat stabil dan berterusan dalam persekitaran. Kehadiran hidrokarbon heterosiklik dalam air laut akibat proses perindustrian dan tumpahan petroleum akan menyebabkan pencemaran alam sekitar marin dan akan menjejaskan kesihatan mamusia. Bioremediasi adalah salah satu penyelesaian untuk degradasi hidrokarbon heterosiklik. Ia melibatkan aktiviti mikroorganisma yang mengoksidakan gelang aromatik hidrokarbon heterosiklik. Degradasi bakteria mengasingkan dari air laut adalah berguna untuk membersihkan hidrokarbon heterosiklik dalam air laut. Kajian ini bertujuan untuk mengasingkan degradasi bakteria hidrokarbon heterosiklik dari pantai Sarawak. Lima bakteria telah diasingkan dan dicirikan melalui ujian biokimia dan teknik molekul. Sifat-sifat kimia bagi setiap bakteria telah diuji bagi menentukan keupayaan degradasi masing-masing. Hasil kajian menunjukkan bahawa bakteria yang diasingkan boleh memainkan beberapa peranan dalam heterocyclic hidrokarbon degradasi.

Kata kunci: Hidrokarbon heterosiklik, bioremediasi, degradasi bakteria, persekitaran marin

1.0 Introduction

Worldwide industrial and agricultural developments have released a large number of natural and synthetic hazardous compounds into the environment due to careless waste disposal, illegal waste dumping and accidental spills. As a result, there are numerous sites in the world that require cleanup of soils and sludge. Heterocyclic hydrocarbons are one of the major groups of these contaminants.

Heterocyclic hydrocarbons are a class of organic compounds that consist of two or more fused aromatic rings with various structural configurations (Bamforth & Singleton, 2005). They are formed by incomplete combustion of organic matter. Heterocyclic hydrocarbons are widely distributed in soils and sediments, groundwater and the atmosphere. In marine environments, sources of heterocyclic hydrocarbons include the human activities like industrial processes such as petrochemical industry, domestic and industrial wastewater, and spillage of petroleum products from ships (Pinyakong *et al.*, 2012). Some examples of heterocyclic hydrocarbons are naphthalene (Nap), phenanthrene (Ph), fluorene (Flu), carbazole (Car), acenaphthylene (Acy), acenaphthene (Ace), anthracene (An), and fluoranthene (Flt).

Being a derivative of benzene, heterocyclic hydrocarbons are thermodynamically stable and widely distributed in the environment. They are highly recalcitrant molecules that can persist in the environment because of their low water solubility. Most heterocyclic hydrocarbons are toxic. In general, the higher the molecular weight of the heterocyclic hydrocarbon molecule, the higher the toxicity, and the longer the environmental persistence of the molecule (Bamforth & Singleton, 2005).

Due to the toxicity of the heterocyclic hydrocarbon, it may affect human health. Some heterocyclic hydrocarbons are carcinogenic to marine organisms and may transfer to

humans through seafood consumption (Geiselbrecht *et al.*, 1998). Bioremediation programs are set up for heterocyclic hydrocarbon degradation. Previous studies have indicated that the important role of bacteria in heterocyclic hydrocarbon degradation. Most degrading bacteria have been isolated from onshore or freshwater sites such as soils, river water, and activated sludge, and thus are not useful for bioremediation in marine environments (Maeda *et al.*, 2009). Although a few degrading marine bacteria have been isolated from marine environments, more information regarding marine degrading bacteria is required to implement effective bioremediation programs in marine environments.

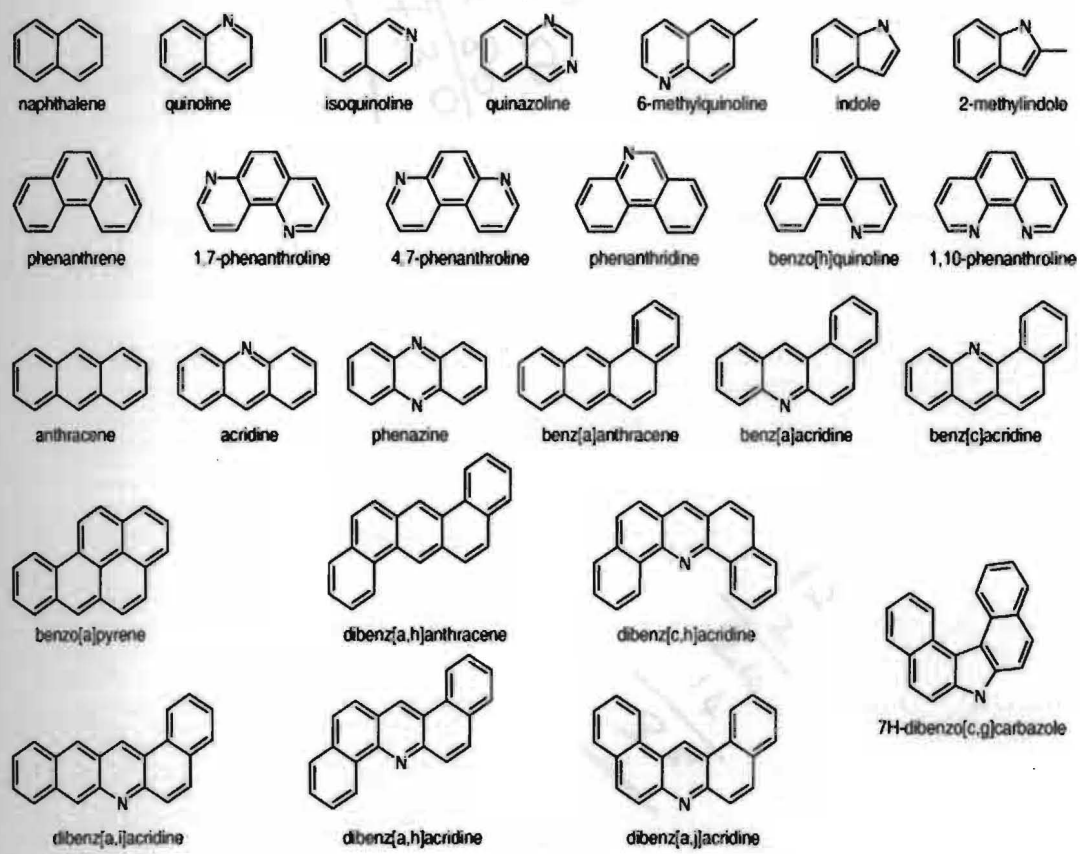
The objectives of this study are:

1. To isolate heterocyclic hydrocarbon degrading marine bacteria from seawater.
2. To characterize the physical and chemical properties of isolated marine bacteria.
3. To identify species using 16S rDNA sequences.

2.0 Literature Review

2.1 Heterocyclic Hydrocarbon

Heterocyclic hydrocarbons are very stable organic molecules that made up of two or more aromatic rings with a pair of carbon atoms shared between rings. They contain only carbon and hydrogen and exist as colorless, pale yellow or white solids. The general characteristics of heterocyclic hydrocarbon are high melting and boiling points, low vapor pressure, and very low water solubility (Katarina, 2011). Due to these characteristics, heterocyclic hydrocarbons are resistant to degradation and can persist in the environment for long period. Some examples of heterocyclic hydrocarbon are shown below:



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2.2 Sources of Heterocyclic Hydrocarbon

There are two major sources of heterocyclic hydrocarbon in the environment: natural and anthropogenic sources. Natural sources include forest and grass fires, oil seeps, volcanoes, chlorophyllous plants, fungi, and bacteria. Anthropogenic sources of heterocyclic hydrocarbon include petroleum, electric power generation, refuse incineration, home heating, internal combustion engines, production of coke, carbon black, coal tar, and asphalt (Katarina, 2011). Heterocyclic hydrocarbons naturally occur in fossil fuels such as coal and petroleum and also formed during the incomplete combustion of organic materials such as coal, diesel, wood and vegetation (Bamforth & Singleton, 2005).

Heterocyclic hydrocarbons are widely distributed in soils and sediments, groundwater and the atmosphere. According to Bamforth and Singleton (2005), heterocyclic hydrocarbons have been detected in marine sediments such as San Diego Bay, California, and the Central Pacific ocean, intertidal sediments, gas works site soils, and sewage sludge contaminated soils, aquifers and groundwater and in atmospheric deposits such as vehicle exhausts fumes. The heterocyclic hydrocarbons are the major cause of environmental pollution and hence many bioremediation programs have been set up to solve the problem.

2.3 Health and Environmental Concerns

Heterocyclic hydrocarbons are strong mutagenic, carcinogenic and toxic that emitted to the air can be transported over long distances before they are deposited with atmospheric precipitation on soils, vegetation or sea and inland water (Maliszewska, 1999). It has long been known that heterocyclic hydrocarbons can have serious deleterious affects to human health. In 1761, the physician John Hill has first recognizes the link between the use of snuff and nasal cancer (Bamforth & Singleton, 2005). Following this discovery, research into the toxic effects that heterocyclic hydrocarbons have upon mammalian health has

continued. Heterocyclic hydrocarbons may create a risk not only to humans but also to all living organisms.

Heterocyclic hydrocarbons will affect the human health include decreased immune function, cataracts, kidney and liver damage, breathing problems, asthma-like symptoms, lung function abnormalities, and repeated contact with skin may induce redness and skin inflammation (Katarina, 2011). For example, Naphthalene can cause the breakdown of red blood cells if inhaled or ingested in large amounts. Moreover, heterocyclic hydrocarbons are potential carcinogens that can produce tumors. Benzo(a)pyrene, a common heterocyclic hydrocarbons, is shown to cause lung and skin cancer in laboratory animals. When ingested, heterocyclic hydrocarbons are rapidly absorbed into the gastrointestinal tract due to their high lipid solubility (Bamforth & Singleton, 2005). In general, the greater the number of benzene rings, the greater the toxicity of the heterocyclic hydrocarbon.

2.4 Bioremediation

Due to the toxicity of heterocyclic hydrocarbons, there is interest in understanding the physicochemical processes and microbial degradation. The biodegradation of heterocyclic hydrocarbons includes the normal processes of the carbon cycle, and removal of pollutants from the environment (Mrozik *et al.*, 2003). The use of microorganisms for bioremediation of heterocyclic hydrocarbons seems to be an attractive technology for restoration of polluted sites.

Bioremediation can be defined as any process that uses microorganisms or their enzymes to degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health or the environment (Vidali, 2001). Bioremediation may be employed in order to attack specific contaminants, such as heterocyclic hydrocarbons that are degraded

by bacteria. Commonly, bioremediation technologies can be classified as *in situ* or *ex situ*. *In situ* bioremediation involves treating the contaminated material at the site while *ex situ* involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation technologies are bioventing, land farming, bioreactor, composting, bioaugmentation and biostimulation. Most bioremediation systems are run under aerobic condition.

According to Mrozik *et al.* (2003), some microorganisms, mainly from the genera *Pseudomonas* and *Mycobacterium*, are found to be capable of transforming and degrading heterocyclic hydrocarbons. These abilities may be useful in removal of heterocyclic hydrocarbons from the environment. The successful application of bacteria to the bioremediation of heterocyclic hydrocarbons-contaminated sites requires a deeper understanding of how microbial heterocyclic hydrocarbons degradation proceeds (Mrozik *et al.*, 2003).

2.5 Degradation of Heterocyclic Hydrocarbon

Microorganisms have been widely used in heterocyclic hydrocarbons degradation. It is because microorganisms have some characteristics that suitable for bioremediation process. Microorganisms can be isolated from almost any environmental conditions. Microbes will adapt and grow at low temperatures, as well as extreme heat, desert conditions, in water, with excess of oxygen, and in anaerobic conditions, with the presence of hazardous compounds or on any waste stream (Vidali, 2001). The main requirements are an energy source and a carbon source.

The basis of microbial metabolism is the oxidation of the aromatic ring, followed by the systematic breakdown of the compound to heterocyclic hydrocarbons metabolites and carbon dioxide (Bamforth & Singleton, 2005). In the presence of oxygen, the

microorganisms undergo aerobic metabolism. Some examples of aerobic bacteria recognized for their degradative abilities are *Pseudomonas*, *Alcaligenes*, *Sphingomonas*, *Rhodococcus*, and *Mycobacterium* (Vidali, 2001). These microbes have often been reported to degrade pesticides and hydrocarbons, both alkanes and polyaromatic compounds (Vidali, 2001). Many of these bacteria use the contaminant as the sole source of carbon and energy. Anaerobic metabolism of heterocyclic hydrocarbons is occurring through the hydrogenation of the aromatic ring.

2.6 Factors Affecting the Bioremediation of Heterocyclic Hydrocarbon

For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. As bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Vidali, 2001). The factors that will affect the bioremediation of heterocyclic hydrocarbon are temperature, pH, oxygen, nutrient availability, bioavailability, and salinity. The solubility of heterocyclic hydrocarbon increases with an increase in temperature, which increases the bioavailability of the heterocyclic hydrocarbon molecules (Bamforth & Singleton, 2005). In addition, oxygen solubility decreases with increasing temperature, which will reduce the metabolic activity of aerobic microorganisms. Bacterial growth was not significantly affected by the pH but the oxygen is required for aerobic heterocyclic hydrocarbon metabolism.

3.0 Materials and Methods

3.1 Sample Collecting

Ten liters of water sample was collected from Asajaya, Sarawak. The sample was obtained and stored in plastic container and transported to the laboratory at room temperature. For long term storage, the water sample was stored in a refrigerated room at 4°C.

3.2 Enrichment Culture and Isolation of Degrading Bacteria

3.2.1 First Enrichment Culture

The 10 l of water sample was undergoing filtration process in order to collect the marine bacteria. The collected marine bacteria were suspended in 10 ml of filtered water sample. Enrichment culture was undertaken in conical flask containing 1 ml of the suspended bacteria, 100 ml of artificial seawater media ONR7a and 0.1 % (w/v) substrate (Fluorene, Dibenzofuran, and Dibenzothiophene) as sole carbon source. The conical flask was incubated at 30 °C and shaken at 200 rpm. Flasks were scored positive or negative on the basis of heterocyclic hydrocarbon degradation. After two to four weeks of incubation, the most flasks that showed color changes indicative of heterocyclic hydrocarbon degradation.

3.2.2 Second Enrichment Culture

For the second enrichment culture, 1 ml of bacteria sample was transfer from the first enrichment culture into another 100 ml of artificial seawater media ONR7a and 0.1 % (w/v) substrate (Fluorene, Dibenzofuran, and Dibenzothiophene) as sole carbon source. The sample was incubated at 30 °C and shaken at 200 rpm for another two to four weeks until the color changes. Subsequent identical transfer of

culture was performed in the respective heterocyclic hydrocarbon containing medium to enrich the bacterial consortium.

3.2.3 Isolation of Pure Colony

After second enrichment, the bacteria culture was inoculated on plates containing marine salts solution ONR7a solidified with 0.8 % agarose. Heterocyclic hydrocarbon was added as the sole carbon source. The plates were incubated at 30 °C for several days. Subculture of bacteria was carried out by using the same media in order to isolate the pure colony. Colonies with a clearing zone on the plates was picked with an inoculating needle and purified by streaking several times onto fresh marine agar plates. The growing bacteria colonies was observed. The pure cultures was obtained for further study

3.3 Characterization of Isolated Bacteria

3.3.1 Morphological Tests

3.3.1.1 Morphological Examination

The color and growth pattern of bacteria on ONR7a agar was examined. Morphological examination is the basis of most examination and preliminary identification of bacteria.

3.3.1.2 Gram Staining

A single bacteria colony from overnight culture was obtained using a sterile inoculating loop and mixed with two drops of distilled water to form a smear on a microscope slide. The smear on the glass slide was covered with few drops of primary stain, crystal violet. After a minute of exposure to the staining solution, the

slide was washed with distilled water. Next, the smear was treated with few drops of gram's iodine and allowed to act for a minute. The slide was washed again with distilled water and then decolorized with 70 % ethanol. After the smear decolorized, the slide was washed with distilled water without any delay. The smear was finally treated with few drops of safranin for 1 minute before rinsing with distilled water. Excess water was removed by blotting paper, dried in air and heated fix before examined under light microscope.

3.3.2 Biochemical Tests

3.3.2.1 Methyl Red Test

A single bacteria colony was inoculated into 5 ml of MR-VP broth and incubated at room temperature for two days. After the incubation, 3 drops of methyl red dropped into the MR-VP culture. Red color change indicates a positive reaction, while yellow color indicates a negative reaction.

3.3.2.2 Voges-proskauer Test

Half of the culture from MR-VP broth was transferred into a sterile bijou bottle before the methyl red test will be conducted. 9 drops of Baritt's A following 3 drops of Baritt's B was added into the culture. Development of a deep rose color within 20 minutes indicates a positive reaction. Contrarily, the absence of rose color indicates a negative reaction.

3.3.2.3 Hydrogen Sulfide Test

A single bacteria colony was taken by using a stabbing needle and stabbed into the SIM agar. The culture was incubated at room temperature for overnight. The production of hydrogen sulfide was determined by observing the formation of black

color within the agar, whereas no formation of blackish in the agar indicates a negative reaction.

3.3.2.4 Motility Test

The growth pattern of bacteria in the SIM agar was examined. A negative reaction was observed when bacteria can only grow along the stabbed line, whereas positive reaction was observed when bacteria are capable of spreading form the stabbed region into the bottom of the agar.

3.3.2.5 Catalase Test

A single bacteria colony was inoculated onto Marine agar and incubated at room temperature for overnight. After the incubation, 3 drops of hydrogen peroxide was added directly onto the colonies. Immediate development of bubble indicates a positive reaction. Contrarily, the absence of bubble indicates a negative reaction.

3.3.2.6 Oxidase Test

A piece of Whatman paper was placed on a petri dish. The paper was moistened with 2 drops of oxidase reagent namely N,N,N,N-tetramethyl-p-phenylenediamine. A single bacteria colony was touched with a toothpick and smeared onto the Whatman paper. Change to purple color within a few seconds indicates a positive reaction, while negative reaction was observed when oxidase reagent remains colorless.

3.3.2.7 Citrate Test

A single bacteria colony was inoculated onto Simmons citrate agar and incubated at room temperature for overnight. Color change of the Simmons citrate to blue

indicates a positive reaction, whereas negative reaction was obtained when the agar remains green.

3.4 Extraction of Bacterial DNA

Total DNA was extracted from isolates grown on marine agar plate using standard protocols. The culture was transferred into Eppendorf/Eppy (1.5 ml microcentrifuge) tube and centrifuged for 30 seconds. After that, the supernatant was removed and the cell pellet was resuspended in 567 µl TE buffer before it mixed well through continuous pipetting or vortexing. 30 µl of 10 % (w/v) SDS and 3 µl 20 mg/ml Proteinase K was added to give final concentration of 100 µl/ml Proteinase K in 0.5 % SDS solution. 100 µl of 5 M NaCl solution was added and mixed well. 80 µl of CTAB/NaCl was added into the mixture and it was mixed well and incubated for 10 minutes in a water bath set at 65 ^{0}C. Equal volume of Phenol/ Chloroform/ Isoamyl alcohol (25:24:1) was added to the mixture after 10 minutes and the solution was vortex briefly and centrifuged for 5 minutes to separate the phases. The viscous and clear supernatant was transferred into a new Eppy tube and the aqueous DNA layer was re-extracted using Chloroform/ Isoamyl alcohol (24:1) and centrifuged for 5 minutes. After centrifugation, the supernatant was transferred into a new Eppy tube and 0.6 volume of isopropanol was added to precipitate the nucleic acid. The tube was inverted up and down slowly until white precipitate appears and the DNA precipitate was pelleted by centrifugation for 30 seconds. The supernatant was removed and the DNA pellet was washed with 200 µl of 70 % ethanol and centrifuged for another 5 minutes at room temperature. It was important to be carefully in removing the supernatant and air-drying the pellet. Finally the dried DNA pellet was dissolved in 25 µl of TE buffer and was stored at 4 ^{0}C for further use.

3.5 PCR Amplification

The partial 16S rRNA gene sequence of isolates was amplified by PCR using universal primers with forward primer and the reverse primer. The Table 1 below show the PCR primer used for the PCR amplification.

Primer Designation	Sequence (5' to 3')	Primer Size (bp)	Expected Amplicon Size
PA (forward)	AGAGTTTGATCCTGGCTCAG	20-mer	1500bp
PH (reverse)	AAGGAGGTGATCCAGCCGCA	20-mer	

Table 1: The nucleotide sequence, size and amplicon of the PCR primers.

The constituents of the PCR reaction mixture show in Table 2. All PCR reagents was mixed gently and briefly centrifuged to collect all drops from wall of tube.

PCR Reagents	Quantity (µl)
10X PCR Buffer	2.50
25 mM $MgCl_2$	1.00
10 mM dNTPs	2.50
25 pmol/µl forward primer (PA)	1.25
25 pmol/µl reverse primer (PH)	1.25
Sterile MiliQ water	14.50
5 U/µl *Taq* DNA Polymerase	0.50
DNA template	1.50
Total final volume	**25.00**

Table 2: 1X reaction mixture for 16S rRNA amplification with PA and PH.